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High Resolution Nuclear Magnetic Resonance Studies of the Active Site of Chymotrypsin

I. The Hydrogen Bonded Protons of the “Charge Relay” System

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High resolution proton nuclear magnetic resonance has been used to observe protons at the active site of chymotrypsin A_8 and at the same region of chymotrypsinogen A. A single resonance with the intensity of one proton is located in the low field region of the nuclear magnetic resonance spectrum. This resonance is observed in H_2O solutions but not in 2H_2O . On going from low to high pH the resonance titrates upfield 3 parts per million in both proteins and has a pK of 7.5. The titration can be prevented by alkylating His57 with either of two active site directed chloromethyl ketones. Using these data the proton resonance has been assigned to a proton in a hydrogen bond between His57 and Asp102. Further confirmation of this assignment lies in the observation of a similar resonance in this same low field region of the nuclear magnetic resonance spectrum of trypsin, trypsinogen, subtilisin BPN' and α -lytic protease all of which have the Asp-His-Ser triad at their active sites.

This proton resonance in chymotrypsin A_8 was used as a probe to monitor the charge state of the active site upon formation of a stable acyl-enzyme analogue N^2 -(*N*-acetylalanyl)- N^1 benzoylcarbazoyl-chymotrypsin A_8 . In this derivative the His-Asp proton resonance titrates from the same low pH end point as in the native enzyme, —18 parts per million, to a new high pH end point of —14.4 parts per million (*versus* —15.0 parts per million in the native enzyme). The difference of 0.6 parts per million in the high pH end points between the native and acyl enzyme is interpreted as supporting the suggestion that a hydrogen bond exists between Ser195 and His57 in the native enzyme and zymogen.

We conclude from these studies that the charge relay system from Asp102 across His57 to Ser195 is intact in chymotrypsin A_8 and chymotrypsinogen A, and that, in the native enzyme, it slightly polarizes Ser195.

1. Introduction

The high resolution X-ray crystallographic studies of several serine esterases, chymotrypsin, trypsin, subtilisin and elastase have shown that the spatial arrangement of the catalytic residues at the active sites of these enzymes is virtually identical (Sigler *et al.*, 1968; Stroud, Kay, Cooper & Dickerson, 1970, *Abst. 8th Int. Cong. Biochem. Switzerland*; Watson *et al.*, 1970; Wright *et al.*, 1969). Each enzyme contains a histidine, serine and aspartic acid residue ordered as shown in Figure 1 and close enough for the indicated hydrogen bonds to exist. The constancy of these three amino acids and their particular spatial arrangement in separate enzymes from such varied sources as mammals and bacteria has generated substantial interest at the level of molecular evolution. But these observations are even more exciting from the

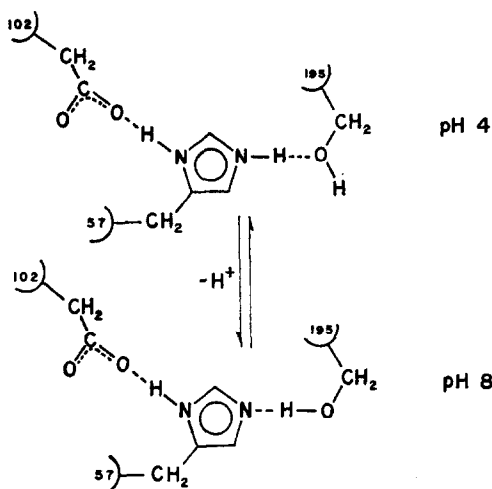


FIG. 1. Proposed hydrogen bond structure at the active site of chymotrypsin A (Blow *et al.*, 1969).

enzymatic mechanism standpoint. Obviously nature has found such a successful mechanism for catalysis that we find it duplicated, to its finest detail, in enzymes across a wide range of species. However, the intriguing question remains as to what features are built into this system which makes it so effective in catalyzing certain reactions? Blow *et al.* (1969) proposed that the linear arrangement of the active site serine, histidine, and aspartic acid functioned to allow a charge polarization at the active site. In this theory the electrons on the buried aspartate are relayed along hydrogen bonds to the serine hydroxyl group helping to make it strongly nucleophilic and thus explaining its reactivity towards ester and amide substrates.

Some experimental evidence has been put forth suggesting that, in the native enzyme, the histidine-serine and histidine-aspartate hydrogen bonds do not exist and, therefore, the "charge relay" system is not intact (Polgar & Bender, 1969). Even if one accepts the existence of the hydrogen bond network which constitutes the charge relay system, its importance in the catalytic mechanism of this enzyme can still be questioned since it appears that catalysis proceeds *via* a concerted general acid-base mechanism rather than *via* stepwise proton transfer (Jencks, 1969).

In recent years high resolution proton n.m.r.[†] has proved very sensitive for studying certain types of hydrogen bonded protons in macromolecules (Sheard *et al.*, 1970). These protons can be observed separately from the bulk of the proton resonances of the macromolecule because they are shifted to lower magnetic fields by the chemical shift of their hydrogen bonds. They are also distinct from the solvent resonance since their exchange with solvent is slowed down by the hydrogen bonds. By observing the n.m.r. resonance of hydrogen bonded protons in chymotrypsin we have attempted to determine:

(1) whether the system of hydrogen bonds proposed by Blow *et al.* (1969) to form a charge relay does exist. (2) Whether polarization of the electronic structure at the active site does occur *via* the hydrogen bonds.

[†] Abbreviations used: n.m.r., nuclear magnetic resonance; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate.

In an earlier communication (Robillard & Shulman, 1972) we presented preliminary proton n.m.r. studies on chymotrypsinogen A and chymotrypsin A_δ in which a single proton resonance was observed at unusually low fields in the n.m.r. spectrum. On the basis of chemical modification results this resonance was assigned to a proton in the active site region of the enzyme hydrogen-bonded between His57 and Asp102 (Robillard & Shulman, 1972). The purpose of these present papers is to present detailed experimental results whose interpretation:

(1) confirms the assignment of the low field proton resonance to a proton hydrogen bonded between His57 and Asp102; (2) supports the proposed hydrogen bond network linking Asp102-His57-Ser195 (the charge relay system); (3) allows us to estimate approximately the charge polarization which occurs *via* this hydrogen bond network.

These data have provided a framework of measurements and interpretation within which one can approach the problem of obtaining a quantitative description of the charge rearrangement and its possible relevance to the enzymatic mechanism.

2. Materials and Methods

(a) *Enzyme samples*

All chymotrypsin samples were prepared from Worthington bovine chymotrypsinogen A (code CGC) or bovine chymotrypsin A_δ (code CDD). The enzyme concentrations and ionic strengths for the individual experiments are stated in the Figure legends.

Bovine trypsinogen (code TG) and trypsin (code TRL) were purchased from Worthington Biochemicals and dialyzed exhaustively at pH 3 and 5°C against 10⁻³ N-HCl. They were then lyophilized at pH 3.

Bovine pancreatic trypsin inhibitor (type 1-P) was purchased from Sigma Chemicals and used without further purification.

Subtilisin BPN' (Nagarse) was purchased from Enzyme Development Corporation 2 Penn Plaza, New York. It was dissolved in 0.01 M-CaCl₂, pH 6, and chromatographed on Sephadex G75 at 5°C in the same salt solution and then lyophilized at pH 6.

α-Lytic protease (myxobacter 495) was a gift of Dr D. R. Whitaker.

p-Nitrophenyl N²-(*N*-acetylalanyl)N¹-benzyl carbazate and *N*-benzyloxycarbonyl-L-glycyl, leucyl, phenylalanyl chloromethyl ketone were synthesized and generously supplied by Dr James C. Powers. Tos-PheCH₂Cl (L-1-tosylamido-2-phenylethyl chloromethyl ketone) was purchased from Cyclo Chemical and used without further purification.

Preparation of Tos-PheCH₂Cl-chymotrypsin A_δ and z-Gly, Leu, Phe-chymotrypsin A_δ from these reagents was accomplished according to the method of Schoellmann & Shaw (1963). The derivatives were chromatographed on CM-cellulose (Wilcox, 1970). Nuclear magnetic resonance spectra were taken on both chromatographed and unchromatographed material. No detectable differences were observed.

(b) *Enzymatic rate assay*

Benzoyl-L-tyrosine ethyl ester rate assay (Worthington Enzyme Manual, 1972) was used to determine the percentage inhibition achieved in preparing inactive alkylated and acylated derivatives. The substrate was purchased from Schwarz-Mann.

(c) *Active site titration*

N-Benzoyloxycarbonyl-L-tyrosine *p*-nitrophenyl ester (Schwarz-Mann) was used as an active site titrant for active chymotrypsin A_δ (Keddy & Kaiser, 1970).

(d) *Nuclear magnetic resonance spectra*

Proton n.m.r. spectra were taken at 220 MHz with a Varian Associates HR220 n.m.r. spectrometer and at 300 MHz on a Varian Associates HR300 operating in the frequency

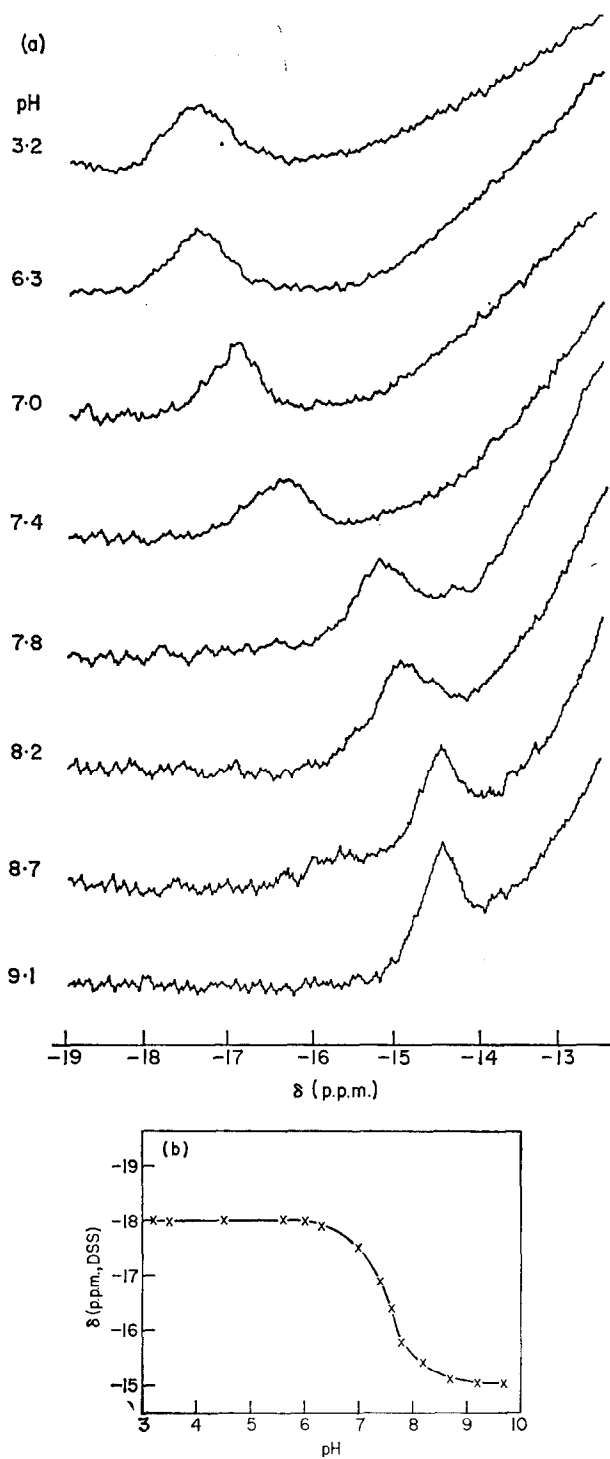


FIG. 2(a). High resolution n.m.r. spectra of chymotrypsinogen A at 3°C. Protein concentrations were 3 to 4 mM in buffered solutions at ionic strength 0.5.

(b) Titration curve of the resonance peaks illustrated in (a).

TABLE I
Resonance linewidth in Hz as a function of pH and temperature for native chymotrypsinogen A,
chymotrypsin A₀ and the acylated and alkylated derivatives

Chymotrypsinogen A		Chymotrypsin A ₀		Acylated chymotrypsin A ₀		Chymotrypsin A ₀		z-Gly, Leu, Phe-chymotrypsin A ₀	
pH	3°C	pH	3°C	pH	3°C	pH	23°C	pH	16°C
	23°C						28°C		3°C
3.5	190	3.5	132	3.3	78	68	86	4.0	77
4.5	200	4.5	144	4.6	78	68	132	5.1	77
5.5	220	5.5	200	5.4	87	100	164	6.0	109
6.5	218	6.5	165	6.5	127	123	191	7.5	113
7.5	196	7.5	—	7.5	95	105	136	8.0	123
8.5	100	8.5	100	8.4	63	64	—	8.5	168
9.5	77	9.5	82	9.5	50	60	—	9.2	86

sweep-mode. The magnetic fields for the resonances are reported in terms of δ parts per million (p.p.m.) downfield from the fields required for the resonance of DSS (2,2-dimethyl-2-silapentane-5-sulfonate). Temperature was maintained constant to ± 1 deg. C with the Varian variable temperature accessory. To improve the signal-to-noise all spectra were an average of 250 to 2000 scans of 50 ms per channel using a Fabri-Tek 1024 computer. The n.m.r. instrument settings for all spectra were as follows: radio frequency field level 40 db (the ethyl benzene quartet begins to saturate at 14 to 15 db), and response 4 Hz. The pH of each sample was measured at room temperature before and after each run. Only those data in which the pH drift was less than ± 0.05 pH unit were used. The pH values referred to in the text are those measured at room temperature. They are not corrected to the temperature at which the spectra were recorded.

3. Results

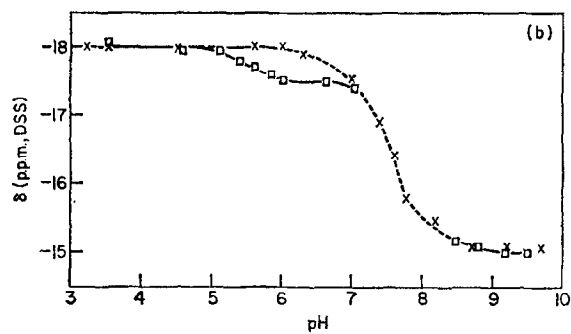
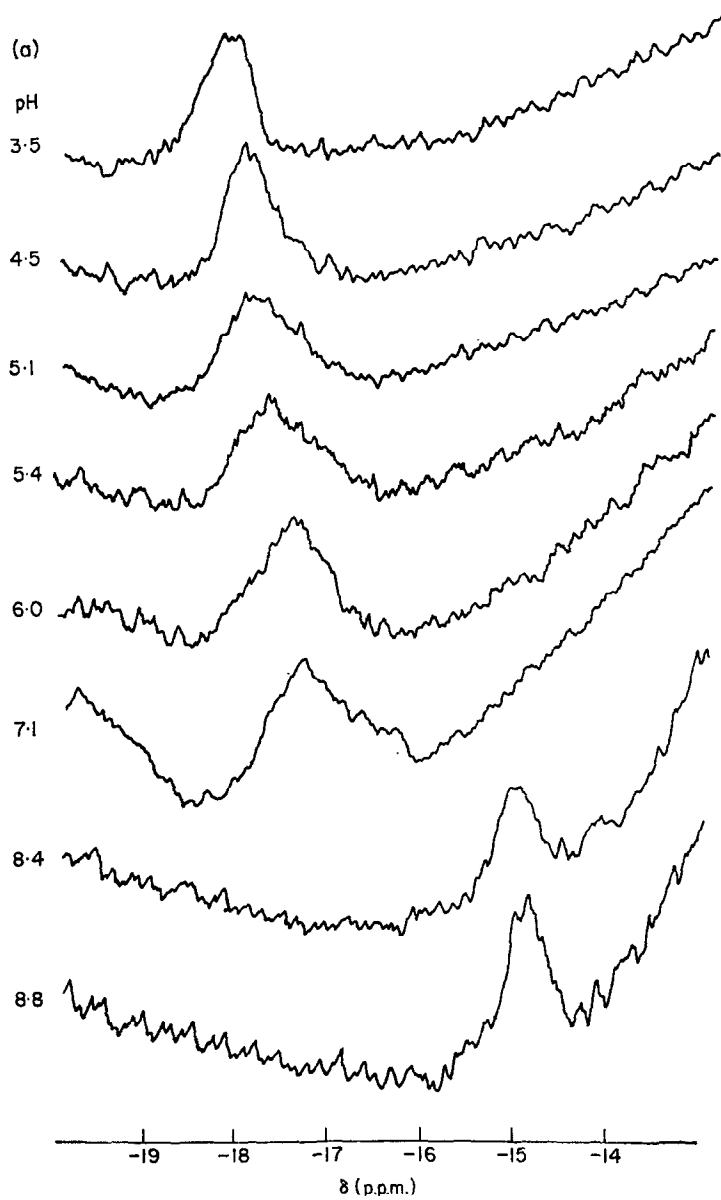
1. Nuclear magnetic resonance spectra of chymotrypsinogen A and chymotrypsin A_δ

Figure 2(a) shows the 220 MHz proton n.m.r. spectra in the low field region as a function of pH for bovine chymotrypsinogen A. The spectra are characterized by a single, broad resonance with an intensity of one proton determined by comparison with a low-field methyl resonance of cyanometmyoglobin. The position of this resonance shifts upfield with increasing pH and the midpoint of the titration is at pH 7.5 at 3°C which will be referred to as the pK . The titration curve is shown in Figure 2(b). The line widths of these resonances in chymotrypsinogen A are shown in Table 1, along with a few widths which could be measured at 23°C. The widths did not arise from slow tumbling caused by aggregation or viscosity because lowering the concentrations by a factor of four did not result in narrower lines. In studying the active enzyme (Fig. 3), we have used chymotrypsin A_δ rather than chymotrypsin A_α because it has less tendency towards aggregation (Miller *et al.*, 1971). Figure 3(a) shows the same low field region of the n.m.r. spectrum for chymotrypsin A_δ as a function of pH. The titration curve for this resonance is superimposed on the chymotrypsinogen A titration curve in Figure 3(b). As can be seen, the general features of the resonance in both proteins are the same. Both start at approximately -18 p.p.m. relative to DSS at low pH and titrate to approximately -15 p.p.m. at high pH with identical pK values. Both proteins show a broadening of the resonance in the middle of the titration curve, however this broadening is more pronounced in the active enzyme and for this reason the resonance cannot be observed between pH 7 and 8. Nevertheless the spectra which have been presented indicate quite clearly in Figure 3(b), that the titration of this resonance in the active enzyme superimposes upon that of the zymogen. The data showing that these two proteins have the same resonance with essentially the same characteristics indicate that the resonances arise from the same proton in each molecule. However, in the active enzyme, there is an additional feature. A second, minor, titration is evident with a pK of 5.5. The total change in the field position during this titration is 0.5 p.p.m. The process responsible for this additional titration is unknown.

The overall titration of both the native enzyme and zymogen as well as the minor titration near pH 5.5 in the active enzyme show a gradual shift of the resonance from one extreme to the other indicating that the two states A and B corresponding

FIG. 3(a). 220 MHz proton n.m.r. spectra of chymotrypsin A_δ. Enzyme concentration was 3 to 4 mM in buffered solutions, $I = 0.5$. The temperature was maintained at +3°C.

(b) Titration curve of the resonance peaks illustrated in (a). (□) Represent the field position of the resonance in (a) and are connected by a solid line. These data are superimposed on the titration curve presented in Fig. 2(b) using (×) and a dashed line.



to the protonated and deprotonated forms are in fast exchange. The criterion for this case (as discussed in Appendix) is that the lifetimes of one of the two states τ_A or τ_B is $\ll [2\pi(\nu_A - \nu_B)]^{-1}$ or 2.5×10^{-4} s where ν_A and ν_B are the n.m.r. frequencies. From the broadening of the chymotrypsinogen resonance in the middle of the titration it is possible to obtain a value of $\tau_A = \tau_B \approx 0.7 \times 10^{-4}$ s at 3°C and pH 7.5 from equation (A3), which is consistent with the fast exchange approximation above.

Loss of active enzyme *via* autolysis occurs when chymotrypsin is kept in solution in the neutral pH range. Competitive inhibitors of the enzyme selectively shift the resonance position of the His-Asp proton and thus it is impossible to protect the enzyme from autolysis using these inhibitors and still observe an unperturbed spectrum (cf. accompanying paper, Robillard & Shulman, 1974). Consequently, spectra of the active enzyme were taken in the absence of competitive inhibitors and the extent of autolysis was determined by activity measurements on duplicate enzyme samples. The results are presented in Table 2. Obviously autolysis does

TABLE 2

Percentage of active enzyme remaining after nuclear magnetic resonance measurements of active chymotrypsin A₀. Activity was determined by benzoyl tyrosine ethyl ester rate assays and compared with a separate sample whose activity and active site concentration were determined at the start of the experiment

pH	% of enzymatic activity remaining relative to the control† at $T = 0$
3	100
4	93
5	85
6	70
7	67
8	61
9	50

† Active site titration of control sample showed 0.86 mol active sites/mol enzyme.

occur during the n.m.r. measurements. However it should be noted that in all cases there is still a concentration of active enzyme 2 mM or greater throughout the measurement. It has been determined in separate experiments that this concentration of enzyme is sufficient to provide a strong n.m.r. signal. Furthermore there is no difference in resonance peak position, line width or intensity from samples with or without 0.1 M-CaCl₂ which stabilizes the enzyme against autolysis. Finally, it might be argued that resonances at pH 8.4 and above in the active enzyme arise from the inactive conformation of the enzyme which results from the ionization of Ile16 ($pK \approx 8.4$) (Fersht, 1972; McConn *et al.*, 1969). This possibility also has been eliminated since these same resonances are present at identical field positions when the enzyme is complexed with *N*-acetyl-L-tyrosine or aniline which stabilize the enzyme in the active conformation (McConn *et al.*, 1968; Hess *et al.*, 1970). These observations, in conjunction with the similarities between the spectra of enzyme

and zymogen, show that autolysis does not affect the n.m.r. spectra which we are observing in the active enzyme.

2. Assignment of the Proton Resonance

(a) Deuterium exchange

When the zymogen or active enzyme is dissolved directly in $^2\text{H}_2\text{O}$, no resonance can be observed in the low field region. The period of time involved in sample preparation and data accumulation is approximately two hours. Therefore the half-life for the exchange process is not greater than one hour and could be considerably shorter. The loss of the proton signal in $^2\text{H}_2\text{O}$ proves that we are looking at an exchangeable proton, one attached to a nitrogen or oxygen. It is this type of proton which is customarily found in hydrogen bonds.

(b) Alkylation

Chymotrypsinogen A and the active chymotrypsin A_δ each have histidine residues at positions 40 and 57. Since the pK of the low field resonance is 7.5 these histidines must be considered as probable sources of the resonance. The only protons on the histidine ring capable of rapid exchange in $^2\text{H}_2\text{O}$ are those associated with the nitrogens at the 1 and 3 positions. Schoellmann & Shaw (1963) and Segal *et al.* (1971) have shown that active chymotrypsin reacts with peptide chloromethyl ketones resulting in an inhibited enzyme in which His57 is alkylated at the N(3) nitrogen. If the proton giving rise to the resonance is located on His57, alkylation of the N(3) nitrogen should produce one of the three results.

(i) The resonance will not be present at any pH

If the proton were located on the N(3) nitrogen the resonance will disappear altogether in the alkylated derivative since the proton will be replaced by the alkyl group.

(ii) The resonance will be present at low pH, but not high pH

If the proton were located on the N(1) nitrogen the resonance in the alkylated derivative could still be present at low pH where the alkyl histidine is protonated. However if the histidine titrates by releasing a proton from the N(1) nitrogen, the resonance would disappear at high pH.

(iii) The resonance will be present at both low and high pH but will not titrate

If the proton were located on the N(1) nitrogen and hydrogen bonded to Asp102, the resonance in the alkylated derivative could be observed over the whole pH range. This requires that the hydrogen bond to Asp102, by locking the proton in place, raises the pK of the alkylated histidine.

Figure 4 shows the low field region of the n.m.r. spectra of the alkylated derivative *z*-Gly, Leu, Phe-chymotrypsin A_δ as a function of pH at 3°C. In both this derivative and Tos-PheCH₂Cl-chymotrypsin A_δ the resonance is still present but it does not titrate in the range of pH 7 to 8. These data suggest that the proton giving rise to the low field resonance is situated on the N(1) nitrogen of His57 and that the pK is raised as in the case (iii) above. Note that if a proton on His40 were responsible for the resonance alkylation of His57 should not generate the results predicted above.

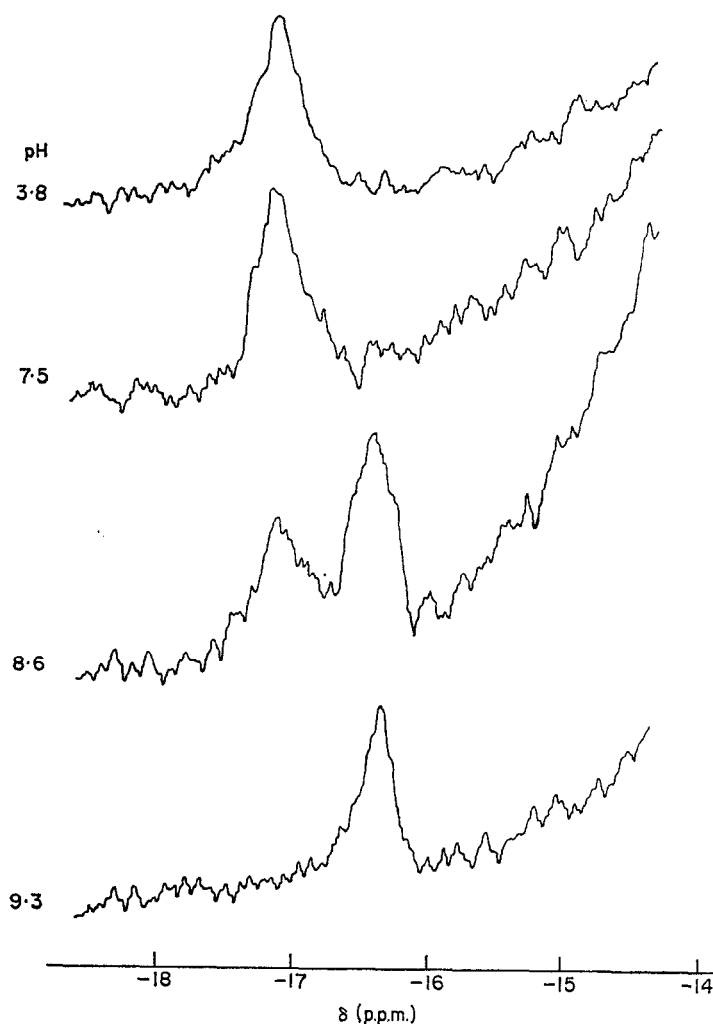


FIG. 4. 220 MHz proton n.m.r. spectra at 3°C of the alkylated derivative α -Gly, Leu, Phe-chymotrypsin A₂. Protein concentration was 2 to 3 mM in buffered solutions, $I = 0.5$. The pH 8.6 spectra is an average of 2169 scans of 25 s duration. The other spectra are an average of approx. 500 scans.

Several other features of these spectra deserve comment. First, alkylation causes a slight shift in the resonance to higher field so that at low pH the resonance is found at -17.3 p.p.m. rather than at -18.0 p.p.m. as in the unmodified protonated enzyme. Second, at low temperature, there are two distinct field positions for the one proton resonance and this position is pH dependent. Below pH 8 the proton resonance is at -17.3 p.p.m. Above pH 9 the resonance is at -16.3 p.p.m. Between pH 8 and 9 the intensity of the -17.3 p.p.m. resonance decreases and that of the -16.3 p.p.m. resonance increases so that at pH 8.4 both resonances are observed and the total intensity of these two resonances together is equal to one proton. This pH dependent shift can

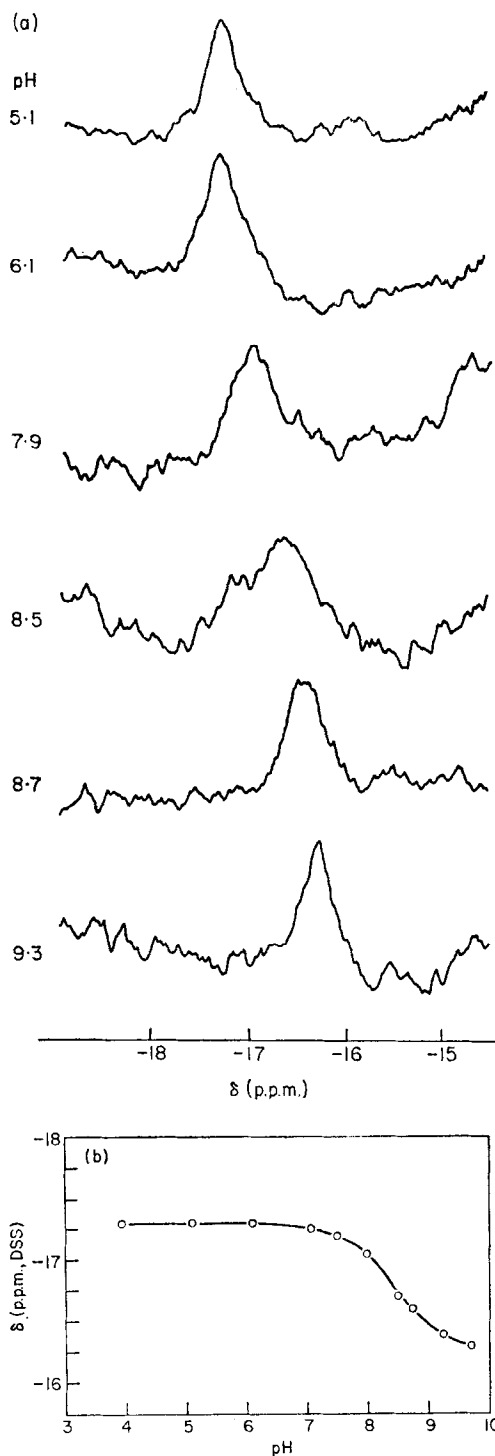


FIG. 5(a). 220 MHz proton n.m.r. spectra at 16°C of the alkylated derivative z-Gly, Leu, Phe-chymotrypsin A₀. Enzyme concentrations were approximately 2 mM in buffered solutions, $I = 0.4$.

(b) Titration curve of the resonances presented in (a).

be analyzed as slow exchange where τ_A and τ_B are $\gg [2\pi(\nu_A - \nu_B)]^{-1}$. Since no broadening or shift of these resonances is observed the lifetimes of the states are long compared to the inverse of the line width, $\tau_A \gg [\pi\delta\nu_A]^{-1}$. The lower limit of the lifetime calculated from the resonance width at half height is $\tau \geq 2 \times 10^{-3}$ s. Clearly we are observing a slow exchange process which might be caused by a pH dependent conformational change in this derivative. By raising the temperature, the characteristics of this resonance are substantially altered. Figure 5(a) presents spectra of the same resonance in the same derivative at 16°C rather than at 3°C as in Figure 4. It is apparent that there is no longer a discrete upfield shift at the higher temperature. Instead the resonance moves gradually upfield with increasing pH, titrating with a pK of 8.4. The resonance width (see Table 1) is narrow at the pH extremes (≈ 80 Hz) and broadens in the middle of the titration (≈ 170 Hz at pH 8.5). Obviously the increase in temperature has taken the exchange rate from the slow exchange limit into intermediate exchange (see Appendix). Since we observe broadening of the resonance during titration we can calculate a more accurate rate constant for the conformational change rather than just the upper limit. The lifetime at either pH extreme is $\geq 1.3 \times 10^{-2}$ s so the rate of exchange between the states at the pK is 7.7×10^3 s $^{-1}$. The titration curve presented in Figure 5(b) again demonstrates that no titration occurs in the range of pH 7.5 in this alkylated derivative. Because of the pH at which this shift and titration does occur one might, at first, relate this conformational change to that which occurs when the α -amino group of Ile16 ionizes. However it has been shown that inhibition of the active enzyme by di-isopropyl-fluorophosphate (McConn *et al.*, 1969) or binding of substrates to the active enzyme (Labouesse *et al.*, 1964) raises the pK of this α -amino group to greater than 10. Therefore when the substrate binding site is occupied, as it is in this present derivative, the conformational change associated with the ionization of Ile16 should not occur below pH 10. Recent X-ray crystallographic studies of subtilisin inhibited with peptide chloromethylketones show that the inhibitor is covalently linked to both the active site histidine and serine residues. Thus, at pH 6, the peptide is bound at the active site as the tetrahedral intermediate (Kraut & Birktoft, personal communication). It is possible that the conformational change being observed in the alkylated chymotrypsin derivative at pH 8.4 arises from a pH dependent dissociation of this intermediate, with the histidine bond remaining intact.

In both *z*-Gly, Leu, Phe-chymotrypsin A $_6$ and Tos-PheCH $_2$ Cl chymotrypsin A $_6$ the low field resonance could be observed. By contrast, in the *N*-methyl derivative it was not possible to observe the resonance. The X-ray crystal structure of the *N*-methyl derivative (Wright *et al.*, 1972) shows a 0.6 Å movement of the N(1) nitrogen of His57 with respect to its position in the native structure. The Asp-His hydrogen bond distance however remains unchanged (2.7 Å). The authors point out that further movement of His57 in the crystal structure is prevented by the C-terminal carboxylate group of Tyr146 of the dyad related molecule which protrudes into the active site. Wright *et al.* (1972) proposed that in solution, where these crystal constraints do not exist, His57 swings out exposing the N(1) nitrogen to solvent and breaking the Asp-His hydrogen bond. Such movements would be consistent with our inability to observe the Asp-His proton resonance. The two alkyl derivatives in which the resonance is observed, on the other hand, are designed to fit snugly into the hydrophobic binding pocket and serve to hold His57 in place.

(c) *Other serine esterases*

Recently Stellwagen & Shulman (1973) were able to assign a number of n.m.r. resonances in horse heart cytochrome *c* by comparing the n.m.r. spectra of several cytochrome *c* variants from other sources. Amino acid deletions or replacements in the variant enzymes, in a number of cases, could be correlated with resonances

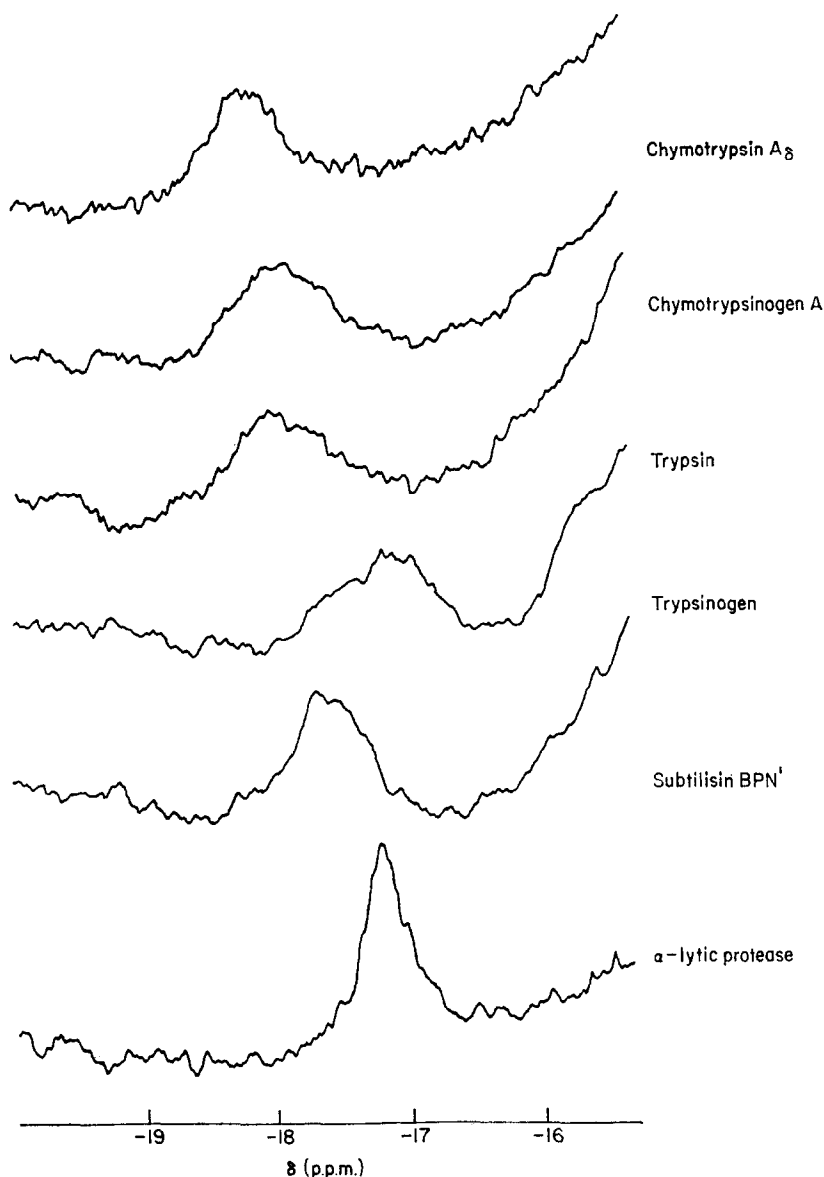


FIG. 6. 220 MHz proton n.m.r. spectra of enzyme samples in H_2O solutions. The concentration of enzyme in each sample was 3 to 4.5 mM determined by absorption at 280 or 282 nm. All spectra were recorded at $3^\circ C$ and are an average of 500 scans each. The specific data for each enzyme sample is as follows:

Chymotrypsin, in 0.25 M-NaCl, pH 3.5; chymotrypsinogen, in 0.25 M-NaCl, pH 3.2; trypsin, in 0.1 M- $CaCl_2$, pH 3.6; trypsinogen, in 0.1 M- $CaCl_2$, pH 3.0; subtilisin, in 0.1 M-N-acetyl-L-tryptophan, pH 6.0; α -lytic protease, in 0.001 N-HCl, pH 3.0.

present in the horse heart enzyme n.m.r. spectrum but absent in the n.m.r. spectra of the variant enzymes. In this section we use the reverse strategy and look for similar resonances among enzymes bearing limited structural homologies.

A number of serine esterases are characterized by the same active site Asp-His-Ser triad found in chymotrypsin. Furthermore the orientation of these residues as well as the distances between them are virtually identical from one enzyme to the next. If the assignment which we have proposed for the low field resonance is correct a similar resonance for the His-Asp proton should be observed in each of the other enzymes which are homologous to chymotrypsin in their active site regions. Figure 6 presents the low field region of the n.m.r. spectrum for H₂O solutions of trypsin, trypsinogen, subtilisin BPN' and α -lytic protease and compares them with the resonance in chymotrypsin A₆ and chymotrypsinogen A. In each enzyme one rather broad resonance is clearly present between -17 and -18 p.p.m. Moreover, in contradiction to recent reports (Hunkapiller *et al.*, 1973) the resonance is present in α -lytic protease where it is in fact most easily observed because of its relatively narrow line width (82 Hz). There are substantial sequence and structure homologies between the chymotrypsin and trypsin enzymes, thus the presence of similar resonances in both enzymes could be attributed to a homologous region. However, between chymotrypsin and subtilisin there are virtually no sequence or structure homologies outside of the active site and substrate binding site regions. Observing a similar resonance in both enzymes supports the proposed assignment. The chymotrypsin, trypsin and subtilisin enzymes each have more than one histidine. Because α -lytic protease possesses only one histidine, the presence of the resonance in this enzyme strongly supports the present assignment.

By itself this series of spectra could not be considered definitive proof of the proposed assignment. However, when combined with the titration data of the native proteins, the deuterium exchange experiment and the His57 alkylation data, all mentioned above, we feel that the assignment of the low field resonance to the His57-Asp102 hydrogen bonded proton is very reliable.

3. The Histidine-Serine Hydrogen Bond

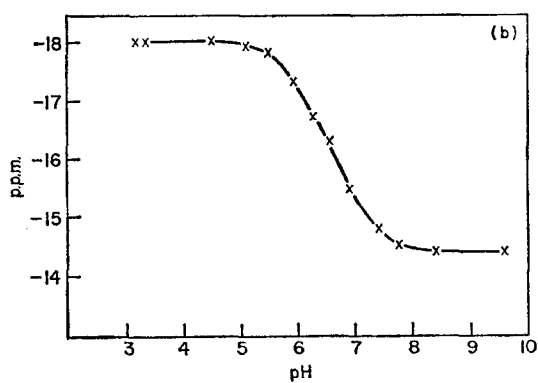
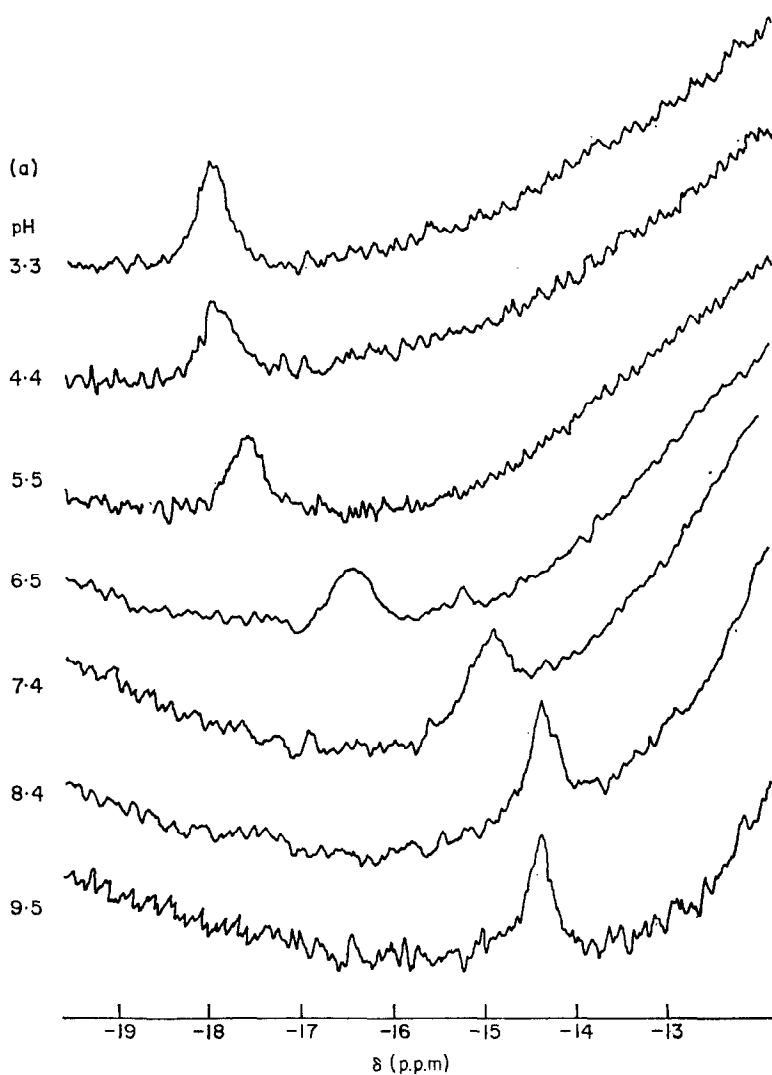
Acylation of serine 195

Experimental evidence on the mechanism of substrate hydrolysis by chymotrypsin using specific and non-specific substrates has established the existence of a transitory acyl enzyme intermediate in the reaction pathway. Since the serine hydroxyl is acylated in this intermediate, substantial changes in the electronic state of the charge relay system at the active site might be expected. True acyl enzymes such as acetyl-tryptophanyl- or acetyl-tyrosyl-chymotrypsin are very unstable and can only be trapped and studied at low pH. The more stable acyl enzyme analogues, suitable for study in the neutral pH region, deviate substantially from the structure of a true acyl enzyme placing, for instance, bulky phosphoryl or sulfonyl groups at the active site.

Our desire to find a stable acyl enzyme analogue which reflected, as accurately as possible the structure of a true acyl enzyme has been realized with the reagent

FIG. 7(a). 220 MHz proton n.m.r. spectra of the acylated derivative N^2 -(*N*-acetylalanyl)- N^1 -benzoylcarbamoyl-chymotrypsin A₆. Protein concentration was approximately 3 mM in buffered solutions, $I = 0.4$ at 3°C.

(b) Titration curve of the resonance peaks illustrated in (a).



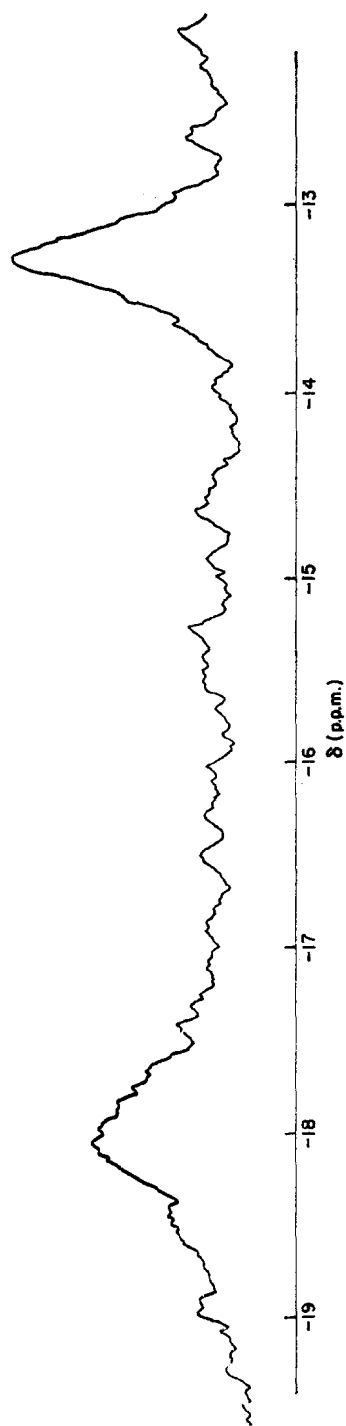


FIG. 8. 300 MHz proton n.m.r. spectrum of chymotrypsinogen A at pH 3.5 at 3°C. The spectrum was taken on a Varian HR300 in the frequency sweep mode using signal averaging to improve the signal-to-noise.

p-nitrophenyl N^2 -(*N*-acetylalanyl) N^1 -benzyl carbazate developed by J. C. Powers (personal communication). This compound reacts with chymotrypsin to form an acyl enzyme analogue which is stable over the pH range 3 to 9, yet it deviates from a true acyl enzyme only in the substitution of a nitrogen for the α -carbon of the phenylalanine residue.

The low field resonance of this derivative was studied as a function of pH. The spectra and titration curve presented in Figure 7(a) and (b) showed several significant differences when compared with those of chymotrypsinogen A and chymotrypsin A₀. First, the *pK* of the resonance changed from 7.5 in the unmodified proteins to 6.5 in the acyl derivative. Second, the total chemical shift between the protonated and unprotonated species (pH 3 to 9) is larger than in either chymotrypsin A₀ or the zymogen. In the zymogen and the active enzyme the resonance titrates from -18.0 p.p.m. at low pH to -15.0 p.p.m. at high pH. The acyl enzyme analogue however, starts off at the same low pH end point of -18.0 p.p.m. but moves to -14.4 p.p.m. at high pH. Hence the acyl enzyme resonance moves 0.6 p.p.m. further upfield at high pH than the same resonance in the native enzyme.

In another stable acyl enzyme, tosyl-chymotrypsin, this resonance was not observed. The X-ray crystallographic structure of this derivative showed that His57 moves outwards into the solvent by approximately 0.3 Å from its position in the native enzyme (Sigler *et al.*, 1968; Henderson *et al.*, 1971). If a similar motion existed in solution it would weaken the His57-Asp102 hydrogen bond and increase the proton exchange rate with solvent water which could explain the disappearance of the n.m.r. peak in this derivative.

A second resonance has been observed in H₂O solutions of chymotrypsinogen A. A spectrum of this resonance and the His-Asp proton resonance taken at 300 MHz is shown in Figure 8. The resonance is observed at -13.3 p.p.m. Its intensity is approximately the same as the low field proton resonance indicating that it corresponds to one proton. However, it can only be observed below pH 4. Apparently the exchange rate of the proton with external solvent increases dramatically over a narrow pH range so that, at pH 4.5 the signal has disappeared. It is significant to note that the -13.3 p.p.m. resonance is not observed in either active chymotrypsin A₀ or the alkylated or acylated derivatives in which the low field resonance does persist. Because the resonance is observed only under such limited conditions no assignment has been possible.

4. Discussion

(a) *Histidine-aspartate hydrogen bonded protons*

The results presented show that the His57-Asp102 hydrogen bond is indeed present and very similar both in chymotrypsin A₀ and in chymotrypsinogen A. Well-resolved resonances arising from ring NH protons of histidine have been observed in water solutions of RNAase A (Patel *et al.*, 1972) and cytochrome *c* (Stellwagen & Shulman, 1973). They occur in the low field region between -10 and -13 p.p.m. relative to DSS. The resonances of carboxylate protons also occur in this same region (Bovey, 1967). The hydrogen bonded proton on the N(1) nitrogen of His57, however, occurs at even lower fields, i.e., -15 to -18 p.p.m. relative to DSS. The abnormally low field position of this resonance is indicative of its environment. Weakly hydrogen bonded protons such as those between carbonyl and amide moieties experience

reasonably small chemical shifts to lower fields on the order of 1 p.p.m. or less. Strongly hydrogen bonded protons such as those between water molecules experience larger shifts on the order of 5 p.p.m. to lower fields (Pople *et al.*, 1959). Since the His57 NH proton resonance is shifted approximately 5 p.p.m. downfield from other histidine NH and carboxylate protons it is clear that this proton, which sits between the N(1) nitrogen of His57 and the carboxylate moiety of Asp102, must be held in a strong hydrogen bond.

It is our long range goal to use these n.m.r. shifts to evaluate the electronic structure of the charge relay system. Clearly it will be desirable to do this in conjunction with calculations of the charge distributions. Two mechanisms are well known to be primarily responsible for downfield shifts of proton n.m.r. lines in hydrogen bonds (Pople *et al.*, 1959). The first is the increased positive charge of the proton which occurs upon hydrogen bond formation and which shifts the resonance further downfield because the nucleus is less shielded. The second is that the neighboring negative ion in the hydrogen bond produces an electric field at the proton which reduces the shielding effect of its electronic cloud and again moves the resonance downfield. The separate contributions of these mechanisms to the downfield shift observed upon hydrogen bond formation and upon protonation of the active site region will be evaluated in a subsequent report.

(b) *The state of ionization of histidine 57*

The pK of an imidazole ring can be a sensitive indicator of its environment. In the past ten years n.m.r. has been used to determine the microscopic ionization constants for individual imidazole rings in macromolecules. In general the pK values are observed over the pH range ~ 5.5 to 7.0 (Meadows *et al.*, 1967; Cohen *et al.*, 1972; King & Roberts, 1971). However pK values as high as 7.6 have been measured (Migchelsen & Beintema, 1973). In each case the transition from imidazolium ion to imidazole is characterized by an upfield shift in the proton resonance. The above mentioned studies measured the proton at the C(2) position of the imidazole ring. More recently, however, histidine titrations in ribonuclease have been measured by monitoring the n.m.r. resonance of a slowly exchanging N-H proton on the imidazole (Patel *et al.*, 1972; Griffin *et al.*, 1973). That N-H proton resonance moves 1.5 p.p.m. upfield during the titration from imidazolium ion to imidazole with the same pK as one of the C(2) protons. The low field resonance assigned to the Asp-His hydrogen bond in this paper responds to changes in pH in a manner similar to the ribonuclease N-H proton, suggesting that His57 is protonated with a pK of 7.5 .

Recent ^{13}C n.m.r. studies have been carried out on α -lytic protease in which the C(2) carbon of the active site histidine was enriched with ^{13}C (Hunkapillar *et al.*, 1973). It should be noted that the ^{13}C n.m.r. spectra were taken at 35°C while our proton n.m.r. spectra were mostly taken at 3 to 5°C . The chemical shift of the ^{13}C resonance titrates over a normal range with a pK of 6.7 but the C-H coupling does not seem to change until pH ~ 3.5 at which point the single pair of resonances split into three pairs. Hunkapillar *et al.* (1973) ignore the titrating chemical shift at pH 6.7 and state that one of the three species observed at pH 3.5 shows that the histidine does not protonate until then. We do not see any change in the His-Asp hydrogen bonded proton resonance in the vicinity of pH 3.5 in chymotrypsin or α -lytic protease. For this reason and by analogy with the ribonuclease results the simplest explanation of our observed shifts would be protonation of His57 with a pK of 7.5 .

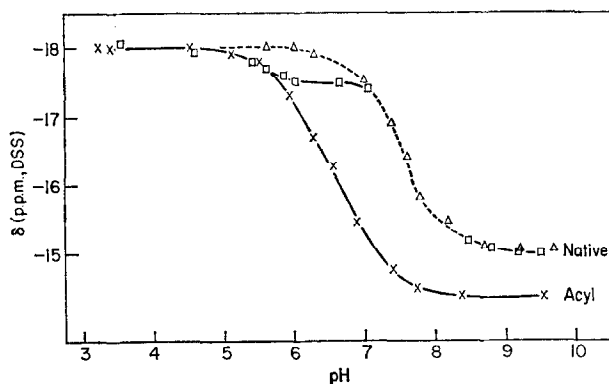


FIG. 9. A comparison of the titration curves for native chymotrypsinogen A and chymotrypsin A_δ and the acylated derivative N^2 -acetylalanyl- N^1 -benzoylcarbamoyl-chymotrypsin A_δ . The data are taken from Figs 2(b), 3(b) and 7(b).

(c) Histidine-serine hydrogen bond

The data obtained from the acyl derivative, N^6 -(N -acetylalanyl) N^1 -benzoyl-carbazoyl-chymotrypsin A_δ , are consistent with the presence of a His57-Ser195 hydrogen bond in the native enzyme and zymogen. This may be seen by comparing the titration of the His-Asp proton resonance in the native and acyl enzymes (see Fig. 9). At low pH His57 is protonated in both the native enzyme and the acyl derivative. As shown at the top of Figure 10 the only slight difference in the two proposed structures is that in the native enzyme the N(3) proton is hydrogen bonded to a serine oxygen. Thus it is not surprising that both resonances occur at the same field, i.e., -18.0 p.p.m. At high pH, however, the proton resonance is 0.6 p.p.m.

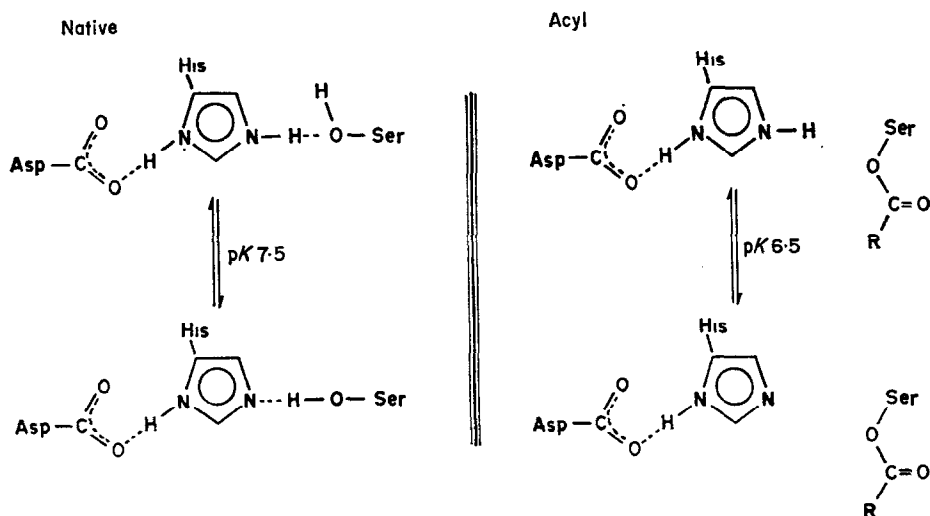


FIG. 10. A comparison of the active sites of active chymotrypsin A_δ and the acylated derivative at high and low pH.

further upfield in the acyl derivative than in the native enzyme or zymogen. The states presumed to be involved are shown at the bottom of Figure 10. In the native enzyme the serine shifts the His-Asp proton resonance downfield whereas in the acyl derivative the serine cannot be directly hydrogen bonded to histidine. Since protonation moves this resonance ~ 3 p.p.m. downfield an internally consistent explanation is that at high pH the His-Asp proton sees a more positive histidine in the native enzyme than in the derivative. Although it would be premature to attempt to describe the charge on the serine oxygen quantitatively from these data, qualitative conclusions can be drawn. First, if, in the native enzyme at high pH, a serine alkoxide ion were formed by completely donating a proton to His57, the His-Asp proton resonance would not titrate, since the histidine would be protonated at both pH extremes. Hence any view of the charge relay system as creating an alkoxide ion is too extreme and not supported by these results. Second, the difference of 0.6 p.p.m. between the high pH states of the two enzymes is small compared to the shift of 3 p.p.m. observed upon protonating the active enzyme. Therefore the serine proton must be only partially transferred to the histidine by the charge relay system.

On the basis of these data we conclude, first, that the charge relay system does exist and second that the charge relay system only slightly polarizes the active site serine. In the future we will attempt to interpret these results quantitatively in order to evaluate their effect upon the overall catalytic activity.

APPENDIX

To the two states A and B we assign resonance frequencies ν_A and ν_B and lifetimes τ_A and τ_B . For the present purposes it is sufficient to distinguish the extreme cases of slow and fast exchange and also to present an expression for line widths in the intermediate exchange case under the assumption of equal population of the two states.

1. Slow Exchange

The criterion for slow exchange is that the lifetimes τ_A and τ_B are large compared to $[2\pi(\nu_A - \nu_B)]^{-1}$. At extremely large values of τ_A and τ_B the linewidth at half height is assumed to reach a limiting value of $\delta\nu_0$. As the lifetimes become shorter, while still satisfying the criterion for slow exchange, the lines broaden and the widths become $\delta\nu_A$ or $\delta\nu_B$. In this case the lifetime of the state is determined by the line width according to the expression

$$\frac{1}{\tau_A} = \pi(\delta\nu_A - \delta\nu_0) \quad (A1)$$

with an analogous expression for state B.

2. Fast Exchange

When either τ_A or τ_B is very small compared to $[2\pi(\nu_A - \nu_B)]^{-1}$ then the signal is shifted to an average frequency

$$\nu = P_A\nu_A + P_B\nu_B, \quad (A2)$$

where P_A and P_B are the populations of the nuclei in the two states. In fast exchange the line widths also become the weighted average of the two states. However for the case of intermediate exchange where the τ 's are short enough to give one averaged, shifted line, but still large enough to contribute to the widths, additional width is

$$\delta\nu - \delta\nu_0 = \tau\pi(\nu_A - \nu_B)^2.$$

This applies for the case of equal populations and lifetimes of states A and B as one would find for instance, half way through a titration curve.

In the present study we know, from the data of Figure 2 for chymotrypsinogen A, that $\nu_A - \nu_B = 3.0$ p.p.m. $= 660$ s $^{-1}$ where A and B are identified with the low and high pH states, respectively. At pH = pK = 7.5 we see from Table 1 that at 3°C.

$$\delta\nu = 200$$
 s $^{-1}$

so that

$$\delta\nu - \delta\nu_0 \approx 100$$
 s $^{-1}$ $= \tau\pi(\nu_A - \nu_B)^2$

or

$$\tau \approx 0.7 \times 10^{-4},$$

where we have assumed that the $\delta\nu_0$ has an average value of ~ 100 Hz whose origin is not understood. It should be noted that the value of τ is close to the value of $\tau \approx 1.5 \times 10^{-4}$ seconds calculated from the 25°C rate constants reported for the base catalyzed proton exchange rate of protonated imidazole (Eigen, 1964; Eigen *et al.*, 1960).

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